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# Activation of Rac1 and RhoA Preserve Corneal Endothelial Barrier Function

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**PURPOSE.** The corneal endothelium is responsible for the correct hydration of the corneal stroma. Corneal endothelial cells have a low proliferative capacity, so preserving their barrier function under suboptimal conditions that cause osmotic imbalance, such as those arising from corneal pathologies, age, cryopreservation, and transplantation, is essential for maintaining corneal transparency. We have investigated the signaling induced by hyperosmotic shock that reversibly disrupts corneal endothelial barriers in human endothelial cells and in murine corneas.

**METHODS.** Endothelial barrier properties were analyzed in vitro by electric cell substrate impedance sensing (ECIS) and confocal microscopy of the human endothelial cell line B4G12-HCEC, and, ex vivo, by confocal microscopy and stimulated emission-depletion (STED) super-resolution microscopy of murine corneas. Cell signaling in response to hyperosmotic stress, induced with an excess of sodium chloride, was investigated in B4G12-HCECs. Rho GTPase activity was detected by pulldown assays with recombinant GST proteins fused to the Rho binding domains of Rho effectors.

**RESULTS.** Hyperosmotic stress increased actin polymerization and activated the Rho GTPases Rac1 and RhoA, but not Cdc42. Rac1- and RhoA-mediated pathway inhibition had a minor effect on barrier disruption but partially delayed barrier reformation after stress withdrawal. In contrast, Rac1 and RhoA activation enhanced constitutive endothelial barrier function and accelerated barrier repair.

**CONCLUSIONS.** Our results indicate that Rac1 and RhoA activation do not mediate stress-induced cell contraction but are endothelial responses that act to restore and maintain barrier homeostasis. Therefore, pharmacological activation of these two GTPases could be a therapeutic strategy for preserving corneal endothelial barrier function.

**Keywords:** barrier function, Rac1, osmotic stress, RhoA, ROCK

The corneal endothelium is a monolayer of hexagonal cells located between the anterior chamber and the corneal stroma. The barrier integrity of the endothelium and its active fluid pump mechanism are involved in maintaining the stromal deturgescence required for corneal transparency.<sup>1-3</sup> Abnormalities in the integrity of the barrier of the corneal endothelium are observed under pathological conditions such as Fuchs' dystrophy and uveitis, wherein they are associated with irreversible corneal swelling, loss of vision, and the need for transplantation.<sup>3,4</sup> Preserving the integrity of the endothelium is also a major concern in ophthalmic procedures such as intraocular surgery and corneal transplantation, because mechanical, inflammatory, and osmotic stress can cause loss of endothelial cells.<sup>5,6</sup> Its role in maintaining corneal deturgescence renders the corneal endothelial monolayer particularly sensitive to osmotic changes, in which the balance between the transcellular transport of water and ions and the paracellular pathway mediated by intercellular junctions seems to be critical

for ensuring correct hydration.<sup>7</sup> Indeed, corneal endothelial cells selectively express protein machinery to prevent osmotic imbalance.<sup>8</sup>

Although the presence of corneal endothelial progenitor cells has recently been described in adults,<sup>9,10</sup> mature corneas have a low proliferative capacity.<sup>10</sup> In this respect, improving endothelial barrier function under homeostatic conditions and in response to stress is crucial for maintaining corneal functionality, even at reduced endothelial cell density. Endothelial barrier function depends on cell-cell junctions, of which tight junctions (TJs) are essential in the corneal endothelium.<sup>7</sup> In addition, tight junctional complexes associate with the perijunctional actin cytoskeleton through linker proteins, such as zonula occludens-1 (ZO-1). Perijunctional actin stabilizes cell-cell junctions, but also mediates cell contraction and junctional disruption.<sup>11</sup>

The Rho family of GTPases comprises more than 20 members, of which RhoA, Rac1, and Cdc42 are the main



regulators of the cell-cell junctions and the underlying actomyosin network. The RhoA-mediated pathway can play opposing functions by increasing the assembly of actomyosin filaments during cell contraction, disrupting cellular barriers, and stabilizing cell-cell junctions in various cell types.<sup>12,13</sup> This pathway is mainly controlled by protein phosphorylation mediated by its effectors, the Rho kinases (ROCKs). ROCKI and ROCKII enhance actomyosin contractility by inactivating myosin phosphatase and promoting myosin light chain (MLC) phosphorylation.<sup>14,15</sup> In addition, Rac and Cdc42 activities are required to maintain barrier integrity<sup>16-19</sup> by mediating the formation of actin filaments that associate with proteins from junctional complexes, such as ZO-1 and  $\alpha$ -catenin at the cell periphery.<sup>20</sup> Tight and adherens junctions form complexes with GTPase exchange factors that locally activate RhoA, Rac, and Cdc42. These interactions not only induce a local increase in F-actin, but also regulate the endocytosis, recycling, and degradation of junctional protein complexes.<sup>21,22</sup> The function of each of these Rho GTPases in regulating integrity of corneal endothelial barriers has not yet been addressed in detail, but the analysis of downstream signaling proteins, such ROCK and nonmuscle myosin II, suggests that at least RhoA is involved in corneal endothelial barrier function.<sup>23-25</sup>

With the ultimate aim of preserving corneal endothelial barrier function, we have investigated signals underlying the endothelial response to stress, and then targeted them with pharmacological inhibitors and activators to investigate their role. We have found that hyperosmotic stress activates RhoA and Rac1 but not Cdc42. The inhibition of pathways controlled by RhoA and Rac1 had a moderate effect on barrier contraction and delayed barrier recovery after the withdrawal of stress. In contrast, pharmacological RhoA and Rac1 activation greatly improved endothelial barrier recovery, which suggests that RhoA and Rac1 activation are major homeostatic responses of these cells by which they try to recover their barrier properties. Thus, in corneal endothelium, RhoA and Rac1 activation increase barrier function and could be therapeutic targets for preserving corneal functionality.

## MATERIALS AND METHODS

### Animals

Three-month-old female CD1 mice were used as a source of corneal tissue. All animal manipulations were carried out in accordance with Spanish (RD53/2013) and European (86/609/ECC) regulations, and were approved by the institutional Animal Care and Use Committee of Centro de Biología Molecular Severo Ochoa and Consejo Superior de Investigaciones Científicas. All efforts were made to minimize suffering of the mice. The authors adhered to the ARVO animal statement.

### Chemicals and Antibodies

RhoA inhibitor (C3-transferase), RhoA/Rac1/Cdc42 activator (Rho activator I), RhoA activator (Rho activator II) were purchased from Cytoskeleton (Denver, CO, USA). ROCK inhibitor (Y27632) and Rac1 inhibitor (Ehop) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-ZO-1 antibody was from Zymed Laboratories (San Francisco, CA, USA), anti-RhoA and anti-ERK1/2 antibodies were from Santa Cruz (Dallas, TX, USA), and anti-Rac1 and anti Cdc42 antibodies were from BD Transduction Laboratories (San José, CA, USA). TRITC-conjugated phalloidin was obtained from Sigma-Aldrich. Peroxidase conjugated affinipure donkey anti-mouse IgG and anti-rabbit IgG were purchased from Jackson Immunoresearch

(West Grove, PA, USA). Alexa 488 donkey anti-rabbit IgG was obtained from ThermoFisher (Waltham, MA, USA).

### Human Corneal Specimens

Human corneal tissue was obtained from remnants of donor grafts after central donor trephination prior to keratoplasty. Donors had no history of eye disease, ocular surgery, or any other systemic disease that might have affected the corneas. Corneal rims were maintained in culture with Tissue C medium at 31°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Endothelial Cell Culture

The human corneal endothelial cell line B4G12 (HCEC) was cultured as described elsewhere.<sup>26</sup> Briefly, cells were grown in human endothelial serum-free medium from Gibco-Thermo Fisher Scientific (Waltham, MA, USA), supplemented with 20 ng/mL of FGF-2. Cells were cultured on plastic dishes precoated with 150  $\mu$ g/mL rat collagen-I, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Human umbilical vein endothelial cells (HUVECs) were obtained and cultured as described.<sup>27</sup> Briefly, HUVECs were obtained by digesting the vein of umbilical cords with 0.1% collagenase at 37°C for 20 minutes (after approval by the donor and the ethics committee). Endothelial cells were collected and grown on fibronectin-coated flasks (10  $\mu$ g/mL; Nunclon, ThermoFisher) with EBM-2 medium supplemented with 2% FBS and endothelial cell growth factor (EGM-2) following the manufacturer's instructions (Lonza, Walkersville, MD, USA). For barrier function assays, HCECs were cultured at confluence for a minimum of 48 hours prior to carrying out the experiments. To attain confluence,  $2 \times 10^5$  cells per well were seeded in the ECIS 8WE10 arrays and in the  $\mu$ -Slide eight-well dishes containing plastic polymer coverslips from Ibidi (Martinsried, Germany), and  $3 \times 10^6$  cells were seeded on 60-mm diameter plastic dishes to perform pulldown assays.

### Osmotic Stress in HCECs and Thrombin Stimulation in HUVECs

Hyperosmotic shock in HCECs was induced as follows. Cells were starved for 1 hour with Hank's balanced salt solution (HBSS) to reduce cell signaling from nutrients and growth factors and to control the pH when cells were manipulated outside of the incubator. Human corneal endothelial cells were either not treated or exposed for 1 to 60 minutes to HBSS supplemented with NaCl at concentrations from 50 to 600 mM, depending on the assay, or to HBSS supplemented with 10% to 20% of mannitol for 1 to 10 minutes. Hypo-osmotic stress was induced by exposing cells to HBSS diluted with 25% or 50% H<sub>2</sub>O for 1 to 10 minutes. To promote the reassembly of TJs after osmotic stress, supplemented or diluted HBSS was replaced with fresh HBSS and incubated for a maximum of 180 minutes. As control experiments to address the efficacy of Y27632 and C3-transferase, HUVECs were seeded at confluence on the  $\mu$ -Slide dishes precoated with fibronectin (10  $\mu$ g/ml) and pretreated, when indicated, with 5  $\mu$ M Y27632 (for 30 minutes) or 0.5  $\mu$ g/ml C3-transferase (for at least 2 hours) before stimulation with 1 U/ml thrombin for 30 minutes.

### Osmotic Stress in Murine Corneas

Dissection and whole-mount staining of mature corneas were performed as follows. Eyes were dissected out and placed into Tissue C medium (TC). After iris and lens removal, corneas were exposed to TC alone or TC supplemented with 225 mM

NaCl, inducing osmotic stress for 2 hours. This excess of NaCl induced reversible barrier disruption without significant cellular damage. Endothelial barrier recovery was measured after replacing hyperosmotic TC solution with fresh TC. Corneas were pretreated with Y27632 (5  $\mu$ M) in TC for 30 minutes before the hyperosmotic shock in order to analyze the effect of ROCK inhibition on TJ disassembly. Y27632 was maintained throughout the experiment to examine its effect during the recovery of endothelial barrier. For RhoA inhibition, corneas were treated with C3-transferase (1  $\mu$ g/mL) for 4 hours before exposure to osmotic stress. To analyze the effect of RhoA signaling inhibition during the recovery of barrier integrity, inhibitors were added in TC after osmotic stress withdrawal and maintained for 8 hours in TC. Experiments with Rho activator I (1  $\mu$ g/mL) and RhoA activator II (5  $\mu$ g/mL) were performed in a similar manner to those described for the inhibitors. Corneas were pretreated with these activators for 2 hours before osmotic stress to analyze their effect on TJ disruption. To analyze their involvement in the endothelial barrier recovery, hyperosmotic TC was replaced with TC containing the activators at the indicated concentrations.

### Measurement of TEER (ECIS Assay)

Human corneal endothelial cells ( $2 \times 10^5$  cells per well) were seeded on eight-well arrays (8WE10) from Ibidi, containing 10 gold electrodes per well. Wells were precoated with rat-collagen-I. Forty-eight to 72 hours later, transendothelial electric resistance (TEER) was measured in the arrays with the 1600R electric cell substrate impedance sensing (ECIS) system from Applied Biophysics (Troy, NY, USA). Each experimental condition was measured in duplicate in each experiment. Before applying the osmotic stress, TEER was measured to verify that the cell monolayer had reached a steady state. Cells were starved with HBSS for 1 hour, which had no significant effect on TEER, and then subjected to stress as described. TEER values were normalized with respect to those obtained before the osmotic shock. During NaCl treatment, TEER was monitored for a maximum of 1 hour and, after stress withdrawal, TEER changes were recorded for 2 hours to analyze junction reformation. The percentage of TEER reduction induced by the different experimental conditions was calculated taking into account the average normalized resistance (NR) for ECIS electrodes containing fully contracted cells (observed in NaCl-treated cells) and the average NR of untreated cells (control confluent monolayers produced an average NR value of approximately 1 at the beginning of the experiment). The percentage of reduction in TEER was calculated as  $[(1 - \text{NR experimental condition}) / (1 - \text{NR NaCl}) \times 100]$ .<sup>27,28</sup>

### Pulldown Assays for Measuring Rho GTPase Activity

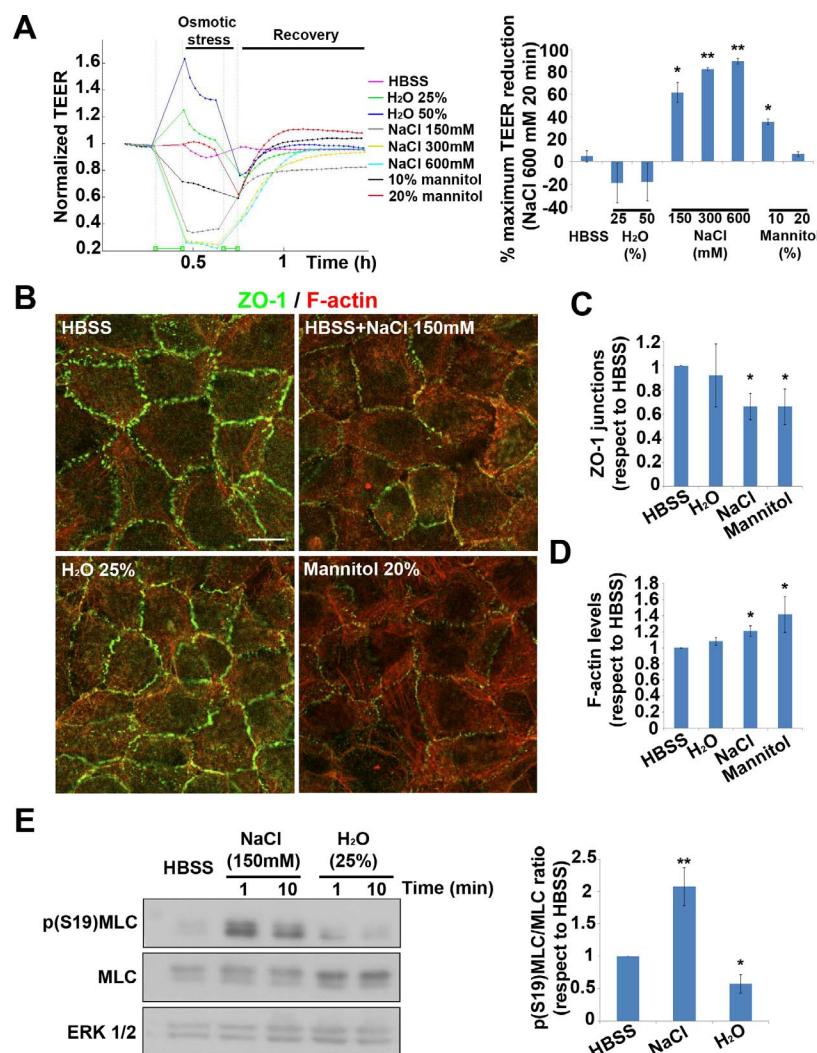
The levels of activated GTP-bound Rho, Rac, and Cdc42 were measured by pulldown assays.<sup>29</sup> Briefly, confluent HBSS-starved HCECs were exposed to the indicated osmotic stress for 0, 2, and 10 minutes. Cells were lysed on ice with 0.5 mL of lysis buffer containing 50 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>, 0.5 M NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA pH 8, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10  $\mu$ g/mL leupeptin, aprotinin, and pepstatin. Two lysates from two culture dishes of 10-cm diameter were pooled per condition. Cell lysates were centrifuged for 5 minutes at 16250g at 4°C. An aliquot of each postnuclear supernatant was kept to measure total Rho protein levels. The supernatants were then incubated with the 10- $\mu$ g recombinant GST proteins previously bound to glutathione-Sepharose beads for 40 minutes at 4°C. Rho proteins bound to recombinant GST proteins were pulled down by centrifugation

for 60 s at 1010g at 4°C. Pulldowns were thoroughly washed with lysis buffer by three additional low-speed centrifugations. Active RhoA was precipitated by pulldown with GST-rhotekin-RBD, a chimeric GST protein containing the Rho binding domain (RBD) of a RhoA effector called rhotekin. Active Rac1 and Cdc42 were isolated by pulldown with GST-PAK-PBD, which contains the Rac1 and Cdc42 (p21) binding domain (PBD) of the common effector p21 activated kinase (PAK)-1. After the pulldown was completed, 10  $\mu$ L of the initial postnuclear supernatants (total Rho proteins) and 10  $\mu$ L of the 30  $\mu$ L of the final pulldown fraction (active Rho proteins) were subjected to SDS-PAGE in 12% acrylamide gels under reducing conditions and transferred to Immobilon-P membranes (Millipore, Darmstadt, Germany). After blocking with 5% nonfat dry milk, 0.05% (vol/vol) Tween20 in PBS, the membranes were incubated with antibodies against RhoA, Rac1, and Cdc42. ERK1/2 was blotted as a loading control. After extensive washes, the membranes were developed using an enhanced chemiluminescence Western blotting kit (ECL; GE Healthcare Life Sciences, Piscataway, NJ, USA). The increase in GTP-loading of Rho proteins with respect to unstimulated cells was determined in the Western blots by calculating the ratio between band intensity in the pulldown lanes (active) and the corresponding band intensity in the lanes containing postnuclear supernatants (total), normalized by the loading controls. Statistical analysis was calculated from a minimum of three independent experiments.

### Immunofluorescence, Confocal Microscopy, and Stimulated Emission Depletion Super-Resolution Microscopy

Corneas, HCECs, and HUVECs were fixed for 1 hour in 4% paraformaldehyde (PFA) and then stored in PBS at 4°C. Cells were permeabilized using 0.2% Triton X-100 for 5 minutes on ice. After blocking with 3% BSA in PBS for 1 hour at room temperature, corneas were incubated overnight at 4°C in blocking solution containing rabbit anti-ZO-1 primary antibody (1:400). For detection, Alexa Fluor-coupled secondary rabbit antibody (1:1000) was used. This was followed by staining for F-actin with TRITC-conjugated phalloidin (1:4000). Tight junctions distribution was measured as the percentage of cell junction area containing ZO-1, using the F-actin staining with phalloidin to detect cellular morphology and total cell-cell junction area. The changes in total F-actin fluorescence intensity during the osmotic stress assays were quantified with the ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) by measuring the raw integrated intensity from confocal images of HCECs stained with TRITC-conjugated phalloidin, taken under the same laser power and gain conditions. An average of 17 cells per image, from eight images taken per experimental condition, performed as technical duplicates (4 images per coverslip) in each experiment, from at least three independent experiments, were analyzed. Perijunctional ZO-1 in murine corneas was quantified from three different images per experiment, each containing an average of 20 cells. Perijunctional ZO-1 was quantified in 10 representative cells per image from a minimum of three independent experiments. Stimulated emission depletion (STED) super-resolution microscopy, was carried out on a Leica TCS SP5 II STED microscope (Leica, Wetzlar, Germany), with STED being used to detect fluorescence from the ATTO-647N fluorophore. Excitation was carried out by a 635-nm pulsed diode laser; de-excitation was done by a MaiTai tunable fs laser at 750 nm. Super-resolution images are presented in inverted gray scale format to ensure the features are displayed clearly.





**FIGURE 1.** Effect of osmotic stress on corneal endothelial barrier function. (A) *Left*: measurements of changes in TEER in confluent HCECs exposed to the indicated HBSS solutions containing the indicated excess of H<sub>2</sub>O, mannitol, and NaCl. These treatments induce reversible changes in barrier function upon 10-minute exposure to osmotic stress. *Right*: quantification of TEER reduction after 10-minute exposure to the indicated osmotic stresses. Maximum TEER reduction (100%) in HCECs was obtained by incubation with 600-mM excess NaCl for 20 minutes. (B) Effect of osmotic stress on the TJ marker ZO-1. (C) Quantification of the junctional area positive for ZO-1 in cells exposed to 25% excess of H<sub>2</sub>O (hypo-osmotic), 300-mM excess NaCl (hyperosmotic), and 20% mannitol (hyperosmotic), normalized with respect to HCECs in iso-osmotic conditions (HBSS). (D) Quantification of F-actin levels in cells exposed to 25% excess of H<sub>2</sub>O, 300-mM excess of NaCl, and 20% mannitol, normalized with respect to HCECs in HBSS. (E) Effect of the indicated osmotic stress on the phosphorylation in Ser19 of myosin light chain (p(Ser19)MLC). *Right graph*, quantification of the ratio between p(Ser19)MLC and total MLC levels detected by Western blot in cells exposed to 10 minutes of the indicated stress in three different experiments. *Graphs* represent the mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.005$ . Scale bar: 10  $\mu$ m.

## Statistical Analysis

The data were summarized as the mean  $\pm$  SEM. Pairs of groups were compared using Student's *t*-test (parametric data). Values of *P* less than or equal to 0.05 were considered to be statistically significant, with critical values indicated as: \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$ . All statistical analyses were performed using SPSS 15.0 for Windows (Armonk, NY, USA).

## RESULTS

### Osmotic Stress Alters the Barrier Properties of Human Endothelial Cells

In order to investigate the signals activated in response to hyperosmotic stress, we exposed confluent monolayers of the HCEC line B4G12<sup>26</sup> to increasing concentrations of mannitol

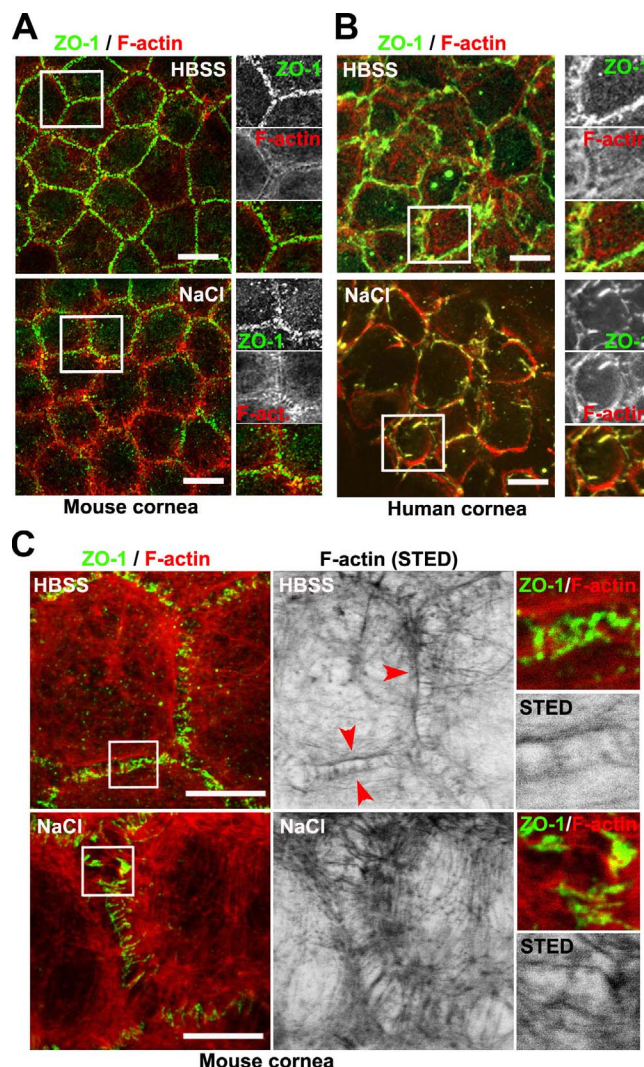
and sodium chloride (NaCl). These treatments provoked a reduction in TEER, measured with an ECIS system, which is indicative of a decrease in endothelial barrier function (Fig. 1A). Conversely, hypo-osmotic stress had an opposite effect and increased TEER. These effects were transient and did not induce significant cellular loss, because TEER values recovered after withdrawal of the osmotic challenge. Parallel confocal analysis of these cells revealed that hypertonicity dispersed the TJ component ZO-1 from cell-cell junctions, whereas hypotonicity did not significantly alter the distribution of this protein (Figs. 1B, 1C). Hyperosmotic stress also increased F-actin levels, suggesting that it affected actomyosin-mediated contraction (Fig. 1D). Nonmuscle myosin II isoforms are protein motors that interact with F-actin and increase actomyosin contractility. Myosin activity is controlled by the MLC, which becomes active upon phosphorylation in a serine residue at position 19. In accordance with the increase in F-actin levels,

MLC phosphorylation, detected by Western blot with an antibody against the phosphorylated (p) S19 of MLC, increased in cells exposed to hyperosmotic stress, but not to hypo-osmotic stress (Fig. 1E).

We performed similar experiments with corneas *ex vivo* to address the effect of hyperosmotic stress in response to salt excess, which occurs *in vivo*, for instance, in the stroma of corneas lacking ion transporters.<sup>8</sup> First, murine corneas were dissected out and exposed to a range of hyperosmotic stress conditions (not shown). Consistent with the reduction in TEER observed in the corneal cell line, the formation of intercellular gaps became evident in murine endothelium exposed to an excess of 225-mM NaCl between 1 and 2 hours, as shown by the ZO-1 distribution (Fig. 2A). ZO-1 was linearly distributed between the two perijunctional actomyosin rings (PAMRs), characteristic of these hexagonal cells, under iso-osmotic conditions (Fig. 2A, enlarged areas). In response to the osmotic shock, the PAMR increased, whereas ZO-1 disappeared in some junctional domains and acquired a more punctate and intense distribution in some others, which overlapped with the F-actin staining (Fig. 2A, bottom images). This overlapping increase in the distribution of the remaining junctional ZO-1 and F-actin was also observed in human endothelium from corneoscleral rims subjected to increasing concentrations of NaCl (Fig. 2B). We then investigated in more detail the changes in F-actin and ZO-1 distribution upon hyperosmotic stress by analyzing the murine corneas by super-resolution confocal microscopy. STED microscopy of the PAMR revealed a perijunctional actin ring formed by bundles of actin filaments (Fig. 2C, arrowheads). Adjacent PAMR appeared connected by F-actin filaments emerging perpendicular to the main actin ring (Fig. 2C, enlarged areas). However, ZO-1 did not colocalize with these F-actin structures. Upon hyperosmotic stress, ZO-1 was dispersed and the PAMR and the perpendicular F-actin connections increased in size and intensity. Interestingly, the ZO-1 clusters that remained at cell borders aligned with these F-actin bundles, suggesting that they mediate the disruption of TJs in response to stress (Fig. 2C, enlarged areas).

### RhoA and Rac1, but Not Cdc42, Are Activated in Response to Hyperosmotic Stress

To further address the cell responses induced by hyperosmotic stress, we analyzed the activity of the Rho GTPases RhoA, Rac1, and Cdc42, which are the master regulators of the actin cytoskeleton and cell barrier function, and are expressed in the mature corneal endothelium.<sup>30</sup> In their active state, most GTPases are loaded with GTP. We examined the GTP-bound state of RhoA, Rac1, and Cdc42, by pulldown assay with recombinant proteins containing Rho-binding domains from Rho effectors that specifically bind these GTP-loaded (active) Rho proteins (Figs. 3A, 3B). We compared the levels of RhoA and Rac1 expression in the cell lysates (Total) with those in the pulldown fractions, which correspond to the GTP-loaded forms of RhoA and Rac1 bound to the recombinant proteins (Active), by Western blot with anti-RhoA and Rac1-specific antibodies. RhoA- and Rac1-GTP levels increased significantly in response to hyperosmotic shock (Fig. 3). In contrast, Cdc42 activation was minor and not statistically significant. We detected an increase in RhoA and Rac1 activity with an excess of NaCl as low as 50 mM (Fig. 3A), although this was more moderate than with an excess of 150-mM NaCl (Fig. 3B). These results demonstrate that hyperosmotic stress activates the signaling pathways controlled by RhoA and Rac1, but not Cdc42, in HCECs.

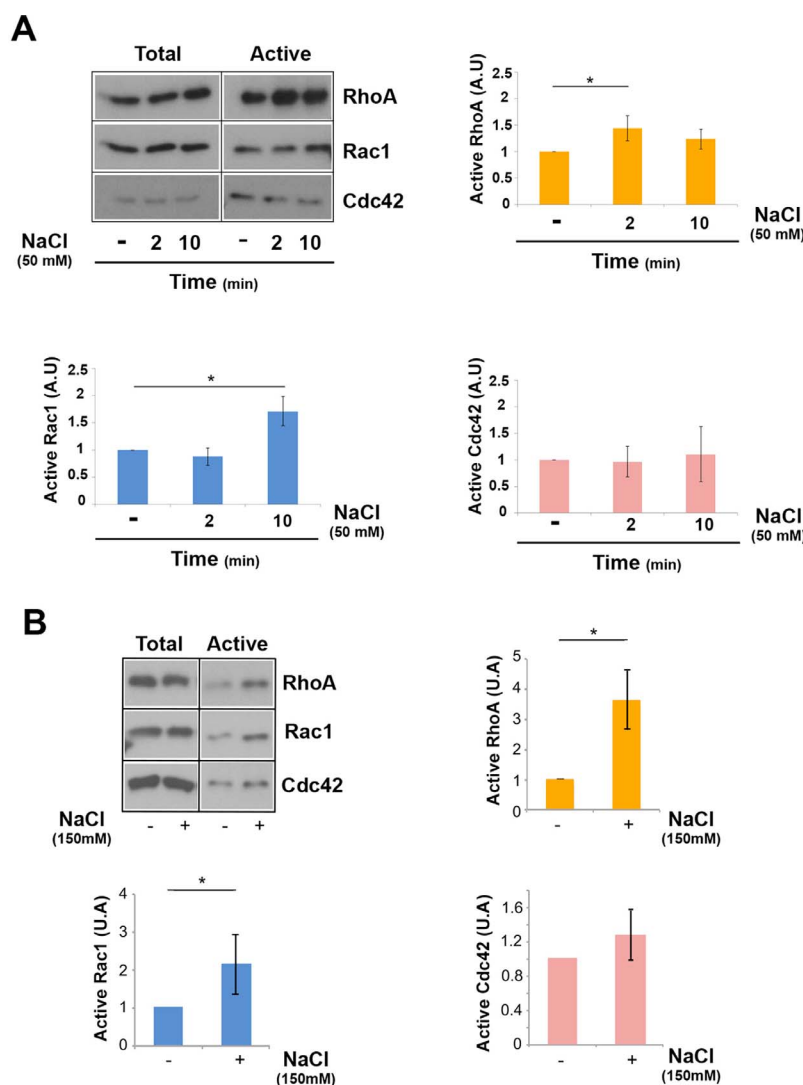


**FIGURE 2.** Effect of hyperosmotic stress in the endothelium of murine and human corneas. (A) Murine adult corneas were dissected out and incubated in HBSS or in HBSS containing an excess of 225 mM NaCl for 1 hour, fixed, and stained for ZO-1 and for F-actin. (B) Human corneoscleral rims were donated after transplantation and incubated in HBSS or in HBSS containing 300 mM NaCl for 1 hour, fixed, and stained for ZO-1 and for F-actin. Scale bars: 20 μm. (C) Imaging TJs and the PAMR by super-resolution confocal microscopy. Dissected murine corneas were incubated in HBSS or in HBSS containing 225 mM NaCl for 1 hour, fixed, and stained for ZO-1 and for F-actin. STED microscopy reveals bundles of actin filaments parallel to the cell border (arrows) and perpendicular actin connections between cells (see enlargements of the STED images). ZO-1 is localized in areas between cells with low F-actin staining (see enlargement of the *squared area* in the ZO-1/F-actin image). With an excess of NaCl, the PAMR and the perpendicular F-actin connections increase in size and intensity and ZO-1 clusters align with the F-actin connections between adjacent PAMRs. *Right images* in each panel correspond to enlargements of the corresponding *squared areas*. Scale bars: 5 μm.

### RhoA and Rac1 Inhibition Do Not Prevent Endothelial Barrier Disruption and Delays Barrier Recovery After Stress Withdrawal

To gain insights into the role of RhoA in disrupting the corneal endothelial barrier after osmotic stress, we pretreated cells with C3 transferase, a specific inhibitor of Rho, conjugated to a permeable peptide.<sup>31,32</sup> C3 transferase



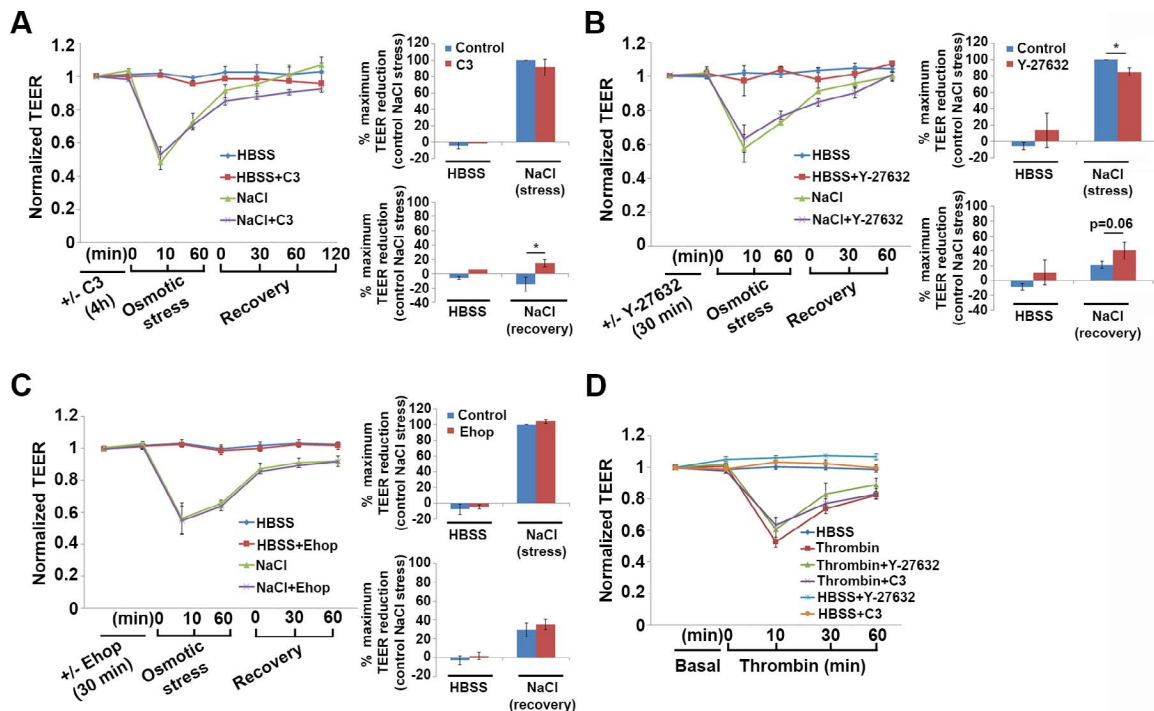


**FIGURE 3.** Hyperosmotic stress activates RhoA and Rac1 but not Cdc42. Pulldown assays of RhoA, Rac1, and Cdc42 in confluent HCEC subjected to hyperosmotic stress of (A) 50-mM excess NaCl for the indicated times, and of (B) 150-mM excess NaCl for 10 minutes. *Graphs* show the quantification of active Rho normalized to total Rho levels from at least three experiments and represent the mean + SEM. \* $P < 0.05$ .

selectively ribosylates RhoA in its effector binding domain, inducing a shift in its molecular weight and inactivating it, as shown by pulldown assay (Supplementary Fig. S1A).<sup>29</sup> C3 transferase did not attenuate TEER decrease during NaCl exposure (Fig. 4A, Supplementary Fig. S1B). However, after stress withdrawal, C3 transferase caused a moderate but statistically significant delay in endothelial barrier recovery (Fig. 4A, Supplementary Fig. S1B). Among the many RhoA effectors, the Rho kinase (ROCK) controls cellular barrier function and mediates actomyosin contractility in different cellular settings.<sup>15</sup> The ROCK inhibitor Y27632, moderately attenuated TEER decrease during hyperosmotic shock and slightly delayed subsequent barrier recovery after the return to iso-osmolality (Fig. 4B, Supplementary Fig. S1C). To address the role of Rac1, we pretreated cells with Ehop-016<sup>33</sup> and analyzed endothelial barrier function. Ehop-016 inhibited Rac1 activity in HCEC exposed to hyperosmotic shock (Supplementary Fig. S1A). However, Ehop-016 modified neither TEER decrease during hyperosmotic treatment, nor barrier function recovery (Fig. 4C, Supplementary Fig. S1D). Pretreatment of HCEC with C3 and Y27632 reduced thrombin-mediated contraction to a lesser extent than what

was previously found in other cell monolayers<sup>27,34</sup> (Fig. 4D). Therefore, although we observed a clear activation of RhoA and Rac1 in response to a hypertonic shock, their inhibition had a minor effect on HCEC barrier disruption. Moreover, inhibiting RhoA-mediated signaling slightly delayed barrier recovery upon stress withdrawal.

To confirm the moderate effect of Rho and ROCK inhibition on barrier reformation, we performed similar experiments with ex vivo murine corneas. These were dissected out and exposed to hyperosmotic stress induced with an excess of NaCl (Supplementary Fig. S2). Although murine endothelium pretreated with RhoA signaling inhibitor (C3 transferase) had lower endothelial F-actin levels than the untreated endothelium before the stress, C3-transferase did not inhibit the increase in the perijunctional F-actin staining occurring in response to the stress (Fig. 5A). Ex vivo barrier reformation assays were performed by exposing the corneas to hypertonic stress and then, when indicated, incubating them with the inhibitors after stress withdrawal (Fig. 5B). This confirmed that osmotic stress did not affect cell viability, because ZO-1 perijunctional staining was fully recovered 8 hours after its removal. We quantified the percentage of ZO-1



**FIGURE 4.** RhoA-ROCK and Rac1 inhibition do not prevent endothelial barrier dysfunction in response to hyperosmotic stress. Human corneal endothelial cells were grown to confluency in ECIS arrays containing gold electrodes and TEER was analyzed by ECIS. TEER was normalized relative to resistance values at the beginning of the assay. Absolute resistance values are shown in Supplementary Figure S1. (A) Effect of C3-transferase (0.5 μg/ml) on barrier contraction induced with 50-mM excess NaCl and the subsequent recovery upon return to iso-osmolarity. (B) Effect of Y-27632 (5 μM) on barrier contraction and the subsequent recovery upon return to iso-osmolarity. (C) Effect of Ehop-016 (5 μM) on barrier contraction and the subsequent recovery upon return to iso-osmolarity. (D) Effect of C3-transferase and Y-27632 on thrombin-mediated barrier disruption. *Graphs* represent the mean + SEM of at least three different experiments. \* $P < 0.05$ .

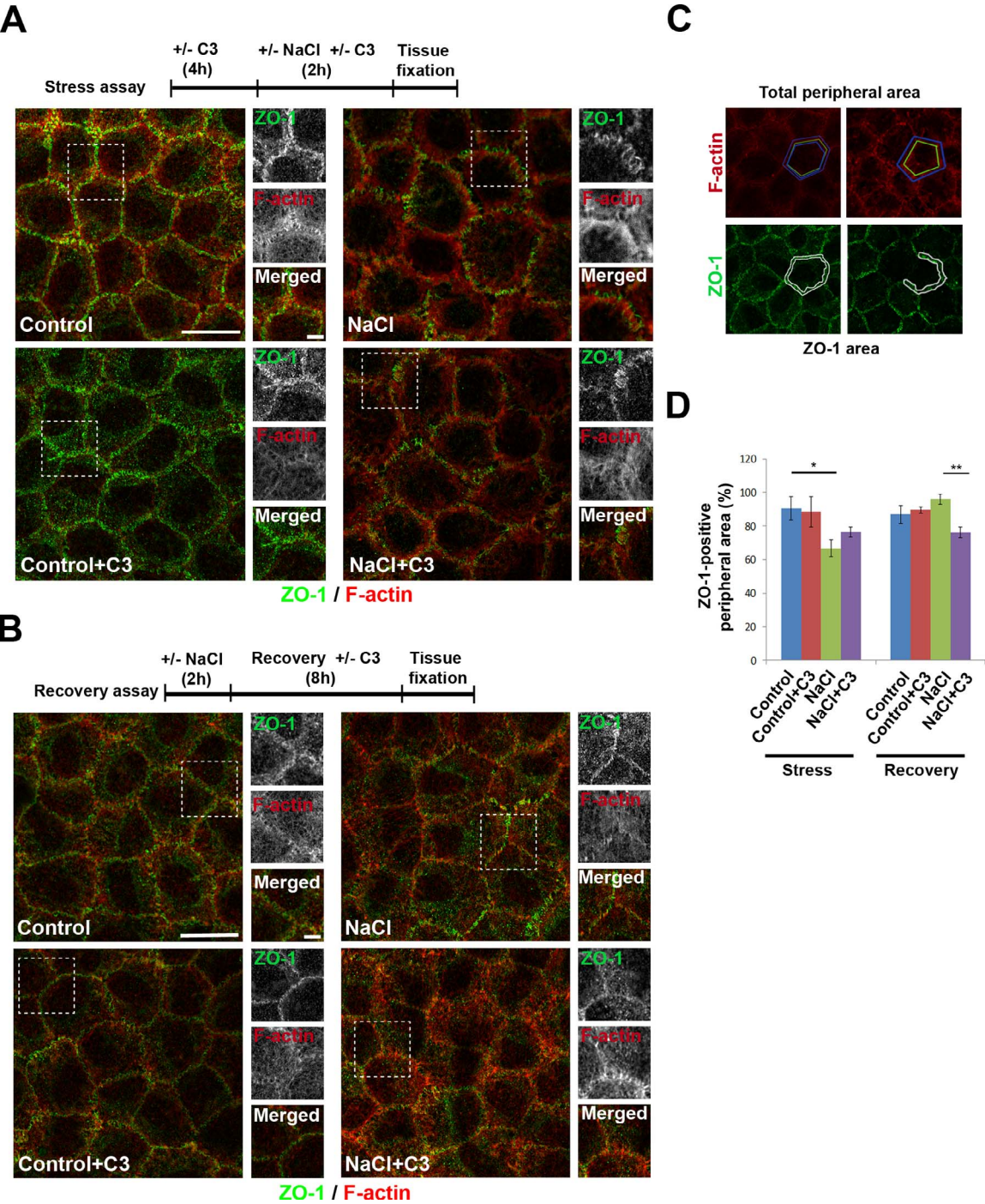
at cell borders in cells exposed or not exposed to stress, as well as in the barrier reformation assays, in the presence or absence of C3-transferase (Figs. 5C, 5D). Quantification revealed that the loss of TJs was not prevented by C3-transferase (Figs. 5A, 5D), suggesting that RhoA is not determinant for barrier disruption. In addition, C3-transferase clearly prevented cell-cell junction recovery (Figs. 5B–D). To determine the role of the RhoA effector ROCK in regulating the endothelial barrier function in murine corneas, we next analyzed the effect of Y-27632 on barrier disruption and recovery following the same quantification procedure (Fig. 5C). Similar to C3-transferase, Y-27632 had no effect on ZO-1 dispersion (Figs. 6A, 6C) but clearly prevented TJ reformation after stress withdrawal (Figs. 6B, 6C). Again, a reduction of F-actin levels in the presence of Y-27632 was observed before but not after, osmotic stress.

Finally, as a positive control of the effect of C3-transferase and Y-27632, HUVECs were stimulated with thrombin in the presence or absence of these inhibitors. Thrombin is a paradigmatic inflammatory mediator that induces F-actin in HUVECs by activating the RhoA-ROCK pathway.<sup>35,36</sup> C3-transferase and Y-27632 clearly reduced the F-actin staining in unstimulated and thrombin-stimulated HUVECs (Supplementary Fig. S3) as previously described.<sup>34,35</sup> Thus, similar to our in vitro results, our findings indicate that RhoA signaling is not required for acute contraction but for the restoration of endothelial barrier properties in murine corneas. RhoA and ROCK inhibitors prevented tight junction reformation, but had no effect on F-actin level increase in response to the osmotic stress in the corneal endothelium.

### Rho and Rac1 Activation Attenuate Endothelial Barrier Disruption and Accelerate Barrier Recovery After Stress

Inhibition of signaling pathways regulated by RhoA affected TJ reformation and increased the dysfunction. We then hypothesized that RhoA and Rac1 activation in response to stress (Fig. 3) are cellular responses that aim to recover the homeostatic state of the endothelial monolayer. Therefore, their activation may favor, rather than undermine, barrier integrity. To address this hypothesis, Rho and Rac1 were activated with two chimeric proteins containing the catalytic domain of a cytotoxic necrotizing factor toxin, conjugated to a penetrating peptide. These catalytic domains block the GTPase activity of various Rho proteins, resulting in constitutively active Rho.<sup>37</sup> Rho activator-I (Act-I) activates RhoA, Rac1, and Cdc42, and Rho activator-II (Act-II) specifically activates RhoA subfamily proteins. Pull-down assays revealed that Act-I activated Rac1 but had little effect on Cdc42 in HCECs (Supplementary Fig. S4). Act-I slightly reduced RhoA activity, probably because this molecule preferentially activates Rac1, which often negatively regulates RhoA function. In contrast, Act-II specifically activated RhoA, but did not induce Rac1 and Cdc42 activity (Supplementary Fig. S4). Human corneal endothelial cells were treated with Act-I and Act-II before osmotic stress. Both activators increased HCEC TEER before the hyperosmotic stress (Figs. 7A, 7B; Supplementary Figs. S5A, S5B) and reduced stress-induced barrier disruption (Fig. 7A; Supplementary Fig. S5A) and recovery (Fig. 7B; Supplementary Fig. S5B). Although the pull-down assays indicated that RhoA was not significantly

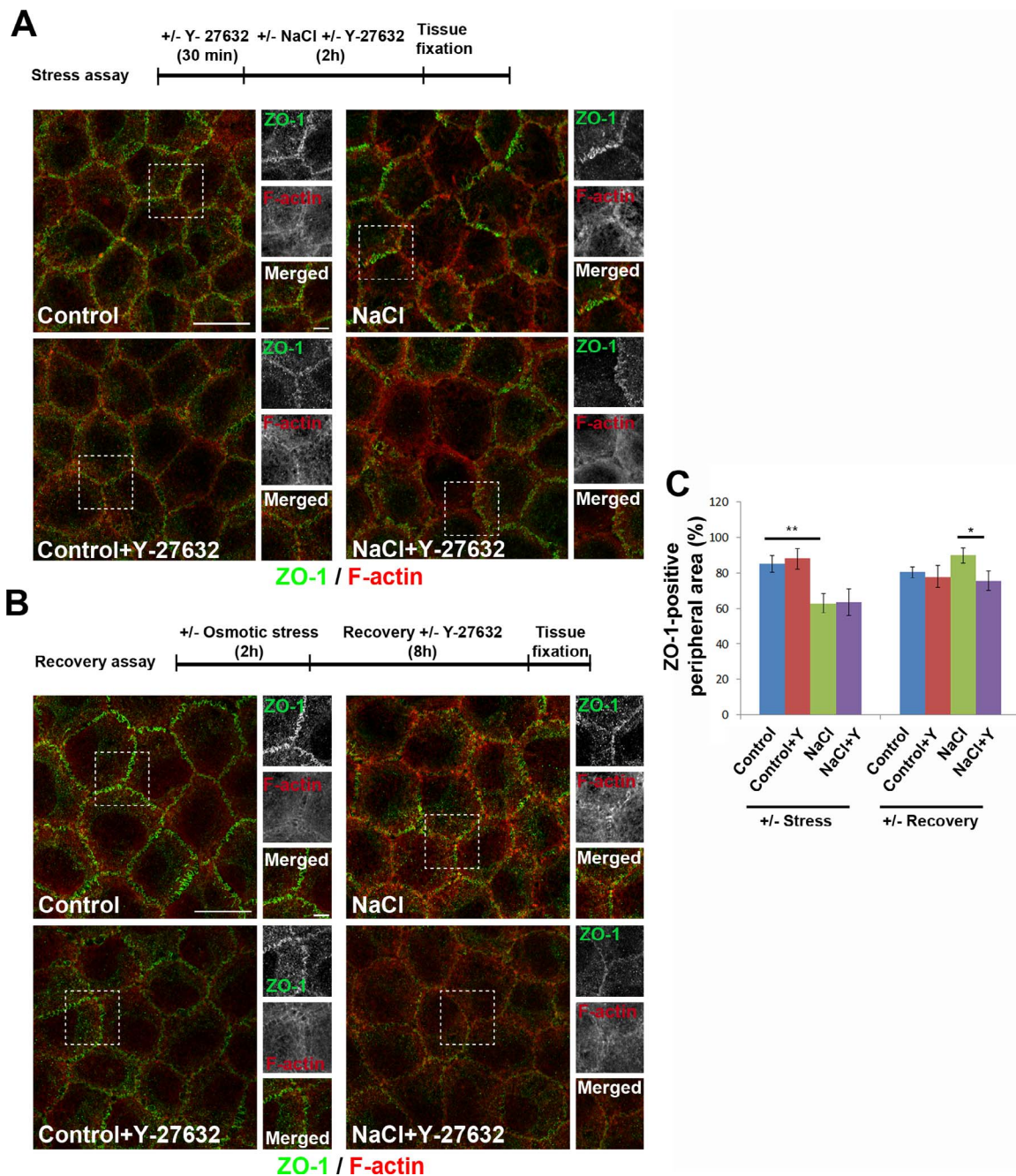




**FIGURE 5.** RhoA inhibition moderately reduces endothelial barrier recovery in murine corneas. Murine corneas were dissected out and subjected to the indicated treatments. **(A)** Effect of C3 transferase during hyperosmotic stress. Murine corneas were pretreated, when indicated, with C3-transferase (1  $\mu\text{g/mL}$ ) and then subjected to hyperosmotic stress. Enlarged details of the *squared areas* are shown to the *right* of the images. **(B)** Effect of C3 transferase during endothelial barrier recovery. Murine corneas were exposed to hyperosmotic stress for 2 hour when indicated (NaCl). After stress withdrawal, corneas were incubated with C3-transferase when indicated. Enlarged details of the *squared areas* are shown to the *right* of the images. **(C, D)** Quantification of the junctional area occupied by ZO-1 from images like those in **(A)** and **(B)**. **(C)** The PAMR was clearly identified with phalloidin-TRITC at cell-cell contacts under all experimental conditions, so the peripheral area of each cell could be determined by drawing a region of interest in ImageJ. The percentage of this area that is positive for ZO-1 staining was then calculated in each treatment. **(D)** Graph represents the mean + SEM of at least three different experiments like those shown in **(A)** and **(B)**. \* $P < 0.05$ ; \*\* $P < 0.01$ . Scale bars: 20  $\mu\text{m}$ . Scale bars in enlarged areas: 4  $\mu\text{m}$ .

activated by Act-I, we also performed experiments with Act-I in the presence of the ROCK inhibitor Y27632, which confirmed that the RhoA pathway does not play a major role in the increase of Act-I-induced barrier function. Importantly,

Act-I and Act-II significantly increased TEER in the absence of any stress, indicating a general and positive effect of RhoA and Rac1 activation on endothelial barrier function. The protective effect on barrier function was also observed in



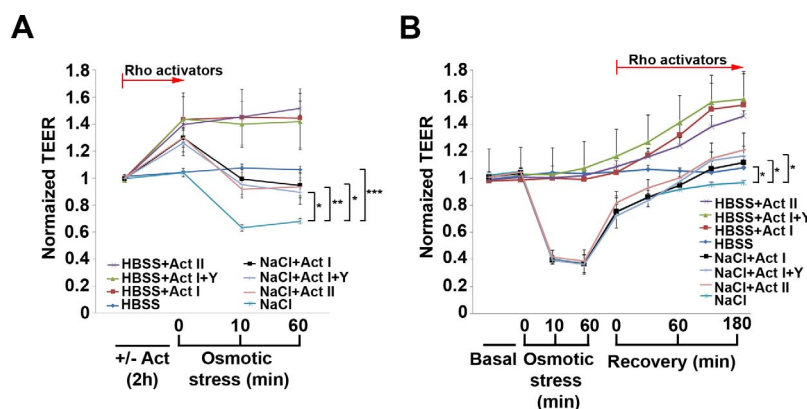
**FIGURE 6.** ROCK inhibition moderately reduces endothelial barrier recovery in murine corneas. Murine corneas were dissected out and subjected to the indicated treatments. **(A)** Effect of Y27632 during hyperosmotic stress. Murine corneas were pretreated, when indicated, with Y-27632 (5  $\mu$ M) and then subjected to hyperosmotic stress. **(B)** Effect of Y27632 during endothelial barrier recovery. Murine corneas were exposed to hyperosmotic stress for 2 hours when indicated (NaCl). After stress withdrawal, the corneas were incubated with Y-27632 when specified. **(A, B)** Enlarged details of the *squared areas* are shown on the *right* of the images. **(C)** Quantification of the junctional area occupied by ZO-1 in the stress and recovery assays was carried out as in Figures 5C and 5D. *Graph* represents the mean + SEM of at least three different experiments \* $P < 0.05$ ; \*\* $P < 0.01$ . Scale bars: 20  $\mu$ m. Scale bars in enlarged areas: 4  $\mu$ m.

murine corneas, in which Act-I and Act-II reduced ZO-1 dispersion in response to hyperosmotic stress (Fig. 8A) and accelerated ZO-1 relocalization at cell-cell junctions during barrier restoration (Fig. 8B), when quantified as in Figure 5C. Interestingly, the effect of Act-I on barrier restoration required the presence of the ROCK inhibitor, suggesting that this activator acts differently on the murine ROCK pathway. Overall, our results indicate that RhoA and Rac1 are good targets for preserving and repairing corneal endothelial barriers under stress.

## DISCUSSION

Rho GTPases play pleiotropic roles regulating the actin cytoskeleton and cell barrier function. In particular, RhoA- and Rac1-mediated pathways can have opposing effects of disrupting and preserving cell barrier function, respectively, and by orchestrating the balance between actomyosin contraction and barrier reformation in various cell types.<sup>19,38-40</sup> At the same time as being active in mediating actomyosin-mediated contraction, the Rho-ROCK pathway is also necessary for the





**FIGURE 7.** RhoA and Rac1 activation preserve endothelial barrier function. Human corneal endothelial cells were grown to confluence in ECIS arrays containing gold electrodes and TEER was analyzed by ECIS. **(A)** Effect of pretreatment with 1  $\mu$ g/ml Act-I (RhoA and Rac1), Act-I + 5  $\mu$ M Y-27632 (Rac1), and 5  $\mu$ g/ml Act-II (RhoA) on barrier integrity. Contraction upon osmotic stress was induced with an excess of 50 mM NaCl. **(B)** Effect of treatment with Act I (RhoA and Rac1 activation), Act-I + Y-27632 (Rac1 activation), and Act-II (RhoA activation) during the return to iso-osmolality (recovery). To better analyze recovery upon stress withdrawal, cells were exposed to osmotic stress with an excess of 150-mM NaCl, which induced greater barrier disruption. TEER was normalized with respect to resistance values before the beginning of the assay. Absolute TEER values are shown in Supplementary Figure S5. *Graphs* represent the mean  $\pm$  SEM of at least three different experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

homeostatic maintenance of cellular barriers.<sup>13</sup> In vitro experiments in monolayers of bovine corneal endothelial cells, in which calcium is removed from the medium to disrupt cell-cell junctions and adhesions, also suggest a dual role for ROCK and actomyosin in corneal endothelial barrier disruption and reformation. On the other hand, the protease thrombin and histamine induce massive RhoA-dependent actomyosin contraction and loss of barrier function in corneal endothelial cells, suggesting that Rho-ROCK mediates acute contraction in corneal endothelium in a comparable way to what occurs in other cell monolayers, such as those of vascular endothelial cells.<sup>25,34,41</sup> Here, we have analyzed the responses of corneal endothelial cells to hyperosmotic stress in vitro and in murine corneas and found that barrier disruption is not prevented by inhibition of the RhoA and Rac1 pathways and that, conversely, Rho-ROCK-MLC and Rac1 activities are required to maintain the endothelial barriers. Our results indicate that this does not occur exclusively in the context of hyperosmotic stress, because MLC inhibition (not shown) and RhoA and Rac1 activation constitutively reduce and enhance, respectively, barrier function. Our pull-down assays suggested that the activation of signaling pathways in response to stress could mediate barrier disruption or, conversely, could constitute a cell response to recover homeostatic barrier function. Our results obtained using inhibitors and activators of these pathways suggest that RhoA and Rac1 activation in response to stress induce signaling pathways to homeostatically maintain corneal endothelial barrier function. Hence, we propose that RhoA-ROCK and Rac1 activation could be strategies for preserving endothelial barrier integrity in response to various types of stress, such as inflammatory, mechanical, and osmotic stress.

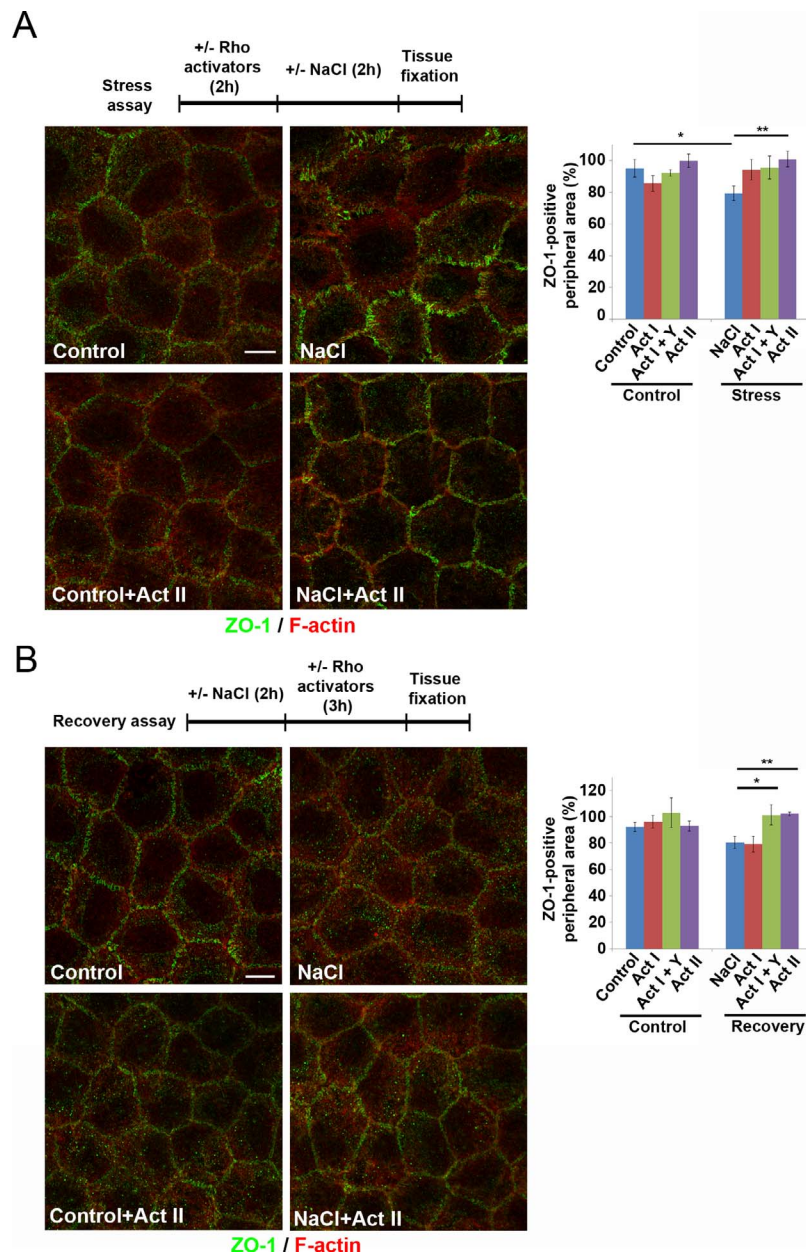
It is of note that in order to promote cell barrier remodeling through cell migration during, for example, corneal wound healing, it is necessary to reduce the stability of cell-cell junctions.<sup>42</sup> In general, the dissolution of cell-cell junctions is associated with increased cell adhesion, migration, proliferation, and cancer.<sup>43,44</sup> Y-27632 is currently being used to promote endothelial cell adhesion and migration for wound closure and to promote cell engraftment during endothelial keratoplasty. Given that ROCK inhibition can have a negative effect on cell migration,<sup>45</sup> the positive effect of ROCK inhibitors in corneal endothelial cells during wound closure<sup>46,47</sup> could be at least partially related to the function of RhoA-ROCK in cell-cell junction formation. Impairment of cell-cell contacts may

have a positive effect on the capacity of individual endothelial cells to adhere to the substratum through focal adhesions, as previously shown in vascular endothelial cells.<sup>44</sup>

Corneal endothelial cells are indeed very sensitive to cell-cell contact inhibition that cause mitotic block. Cell-cell junctions have been perturbed with trypsin EDTA for short periods to unlock this mitotic block and try to promote corneal endothelial cell proliferation in the presence of growth factors. However, this treatment causes epithelial-mesenchymal transition and endothelial cells acquire a fibroblastic phenotype as a consequence.<sup>48</sup> An alternative and elegant strategy to expand corneal endothelial cell cultures consisted of knocking down p120 catenin, a protein from cell-cell junctions, which induced the activation of the RhoA-ROCK-BMP pathway, thereby reprogramming HCEC to act as neural crest-like progenitors. This treatment allowed expansion of HCEC progenitors until p120-catenin levels were recovered, the RhoA-ROCK signaling ceased and HCECs reverted to their original endothelial phenotype.<sup>48,49</sup> Here, we show that hyperosmotic stress recapitulates some of the main requirements for HCEC expansion: reversible cell-cell junction disruption and RhoA-ROCK activation. In addition, active RhoA and Rac1 participate in noncanonical Wnt signaling, which is involved in actin remodeling, development, and epithelial-mesenchymal transition.<sup>49,50</sup> Thus, it seems plausible to hypothesize that hyperosmotic stress may have an effect on corneal endothelial cell proliferation and differentiation.

The paracellular permeability regulated by cell-cell junctions is essential in the "pump-leak" model proposed to explain the control of stromal hydration by the corneal endothelium.<sup>51</sup> The hydrophilic nature of stromal glycosaminoglycans as well as the IOP from the aqueous humor induce a paracellular passage of liquid toward the stroma that is counter balanced by the protein machinery of transporters at the plasma membrane of endothelial cells, which returns ions and water to the aqueous humor.<sup>51,52</sup> When leakiness is excessive, the pumping mechanisms are not sufficient to prevent corneal swelling and corneal transparency is reduced. Mutations in some of these endothelial transporters, such as SLC4A11, cause corneal osmotic imbalance, congenital hereditary endothelial dystrophy and Fuchs endothelial corneal dystrophy.<sup>8,53,54</sup> It is of note that the corneas from the *slc4a11* knockout mice increase NaCl concentration in the stroma, leading to the formation of salt crystals.<sup>8</sup> This may be an





**FIGURE 8.** RhoA and Rac1 activation preserve barrier function in murine corneas. Murine corneas were dissected out and subjected to the indicated treatments. **(A) Left images.** Representative images of ZO-1 and F-actin staining in corneal endothelium subjected to the indicated treatments in a hyperosmotic stress assay (225-mM excess NaCl). **Right graph.** Quantification of the junctional area occupied by ZO-1 in corneal endothelia subjected to iso-osmotic (Control) or hyperosmotic (NaCl) treatments in the presence or absence of Act I (RhoA and Rac1 activation), Act I + Y-27632 (Rac1 activation), and Act II (RhoA activation). **(B) Left images.** Representative images of ZO-1 and F-actin staining in corneal endothelium first subjected to iso-osmotic (Control) or hyperosmotic treatments, and then incubated with iso-osmotic Tissue C to promote barrier recovery (NaCl). **Right graph.** Quantification of the junctional area occupied by ZO-1 in corneal endothelia after the barrier recovery assay in the presence or absence of Act I (RhoA and Rac1 activation), Act I + Y-27632 (Rac1 activation), and Act II (RhoA activation). Scale bar: 15  $\mu$ m. Graphs represent the mean  $\pm$  S.E.M. of at least three different experiments. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

extreme example of osmotic imbalance, but illustrates the fact that dysfunction of the pump-leak equilibrium in corneal pathologies may lead to osmotic stress, which, according to our results, could induce negative feedback by causing additional disruption of the endothelial barrier. Small hypertonic perturbations caused by excess NaCl are compensated by transendothelial fluid transport.<sup>55,56</sup> We show that crossing a certain threshold of hyperosmolarity has a clear effect on tight junctions and paracellular barrier function. Barrier dysfunction upon hyperosmotic shock may occur not only in

corneal dystrophies, but also in the manipulation of corneas during keratoplasty procedures, in which the endothelial cells and the anterior chamber are subjected to acute osmotic and mechanical shock. In conclusion, our results demonstrate the activation of RhoA and Rac1 in response to a stress stimulus that disrupts the endothelial cell-cell junctions. This activation has a positive effect on corneal endothelial barrier function and places these two Rho GTPases at the center of pharmacological strategies for preserving the integrity of the corneal endothelium.

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